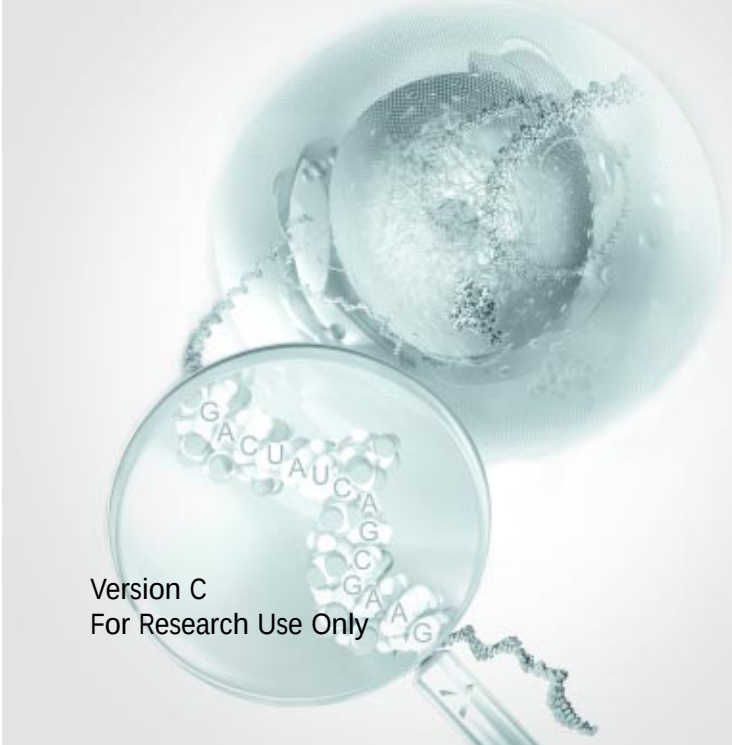
User Guide

RiboAmp™
RNA Amplification Kit

Catalog # KIT0201



ARCTURUS
SYSTEMS FOR MICROGENOMICS

Version C
For Research Use Only

VI. Appendices

Legal Notices

Warranty

Arcturus Engineering, Inc. warrants that the products described in this manual meet the performance standards described in literature published by the company. If a product fails to meet these performance standards, Arcturus will replace the product or issue credit for the full purchase price, including delivery charges. Arcturus provides no other warranties of any kind, expressed or implied. Arcturus' warranty liability shall not exceed the purchase price of the product and shall not extend to direct, indirect, consequential or incidental damages arising from the use, results of use, or improper use of its products. The RiboAmp™ RNA Amplification Kit is intended for research purposes only and is not intended for diagnostic or other purposes.

I. Introduction

A. Background

The RiboAmp™ RNA Amplification Kit enables the production of microgram quantities of antisense RNA (aRNA) from nanogram quantities of total cellular RNA . Small sample collection methods, such as needle aspiration and Laser Capture Microdissection (LCM) limit starting quantities of RNA available for amplification. Microarray hybridization experiments require microgram quantities of probe per array to enable detection, and most studies entail multiple experiments.

The RiboAmp Kit achieves high yields of aRNA with a proprietary linear amplification process (patent pending) using double-stranded cDNA as template in a T7 RNA polymerase-catalyzed amplification. This amplification process yields highly reproducible results through an optimized system of reagents, purification devices, and protocols. The resulting aRNA is suitable for use in RT-PCR, quantitative real-time PCR (Q-RT-PCR), and microarray hybridization experiments.

The RiboAmp RNA Amplification Kit produces unlabeled aRNA that can then be processed using reverse transcription based methods of label incorporation to yield labeled cDNA for hybridization onto expression microarrays. Generation of labeled aRNA for GeneChip® Probe Arrays (Affymetrix) requires modifications of the RiboAmp Kit protocol. (See Appendix F).

The RiboAmp Kit contains a complete set of reaction reagents, nucleic acid purification columns, a control RNA sample, and this user guide. Reagents and materials are supplied for ten, one-round amplifications or five, two-round amplifications. The kit provides premixed enzymes and buffers to save time and increase ease of handling. The protocol is streamlined to enable fast processing while generating reproducible results.

The RiboAmp Kit generates aRNA product that is shorter than the starting mRNA template. The bulk of the aRNA product is 250–1800 bases in length after one round of amplification and

⚠ *Please read this entire manual prior to performing amplifications.*

slightly shorter, under 200 to over 600 bases, after a second round. Messenger RNA makes up an estimated one- to five-percent of total cellular RNA. One round of amplification typically yields up to 1,000-fold amplification of the mRNA, while two rounds may yield up to one-million fold amplification of the mRNA. Amplified aRNA produced using the RiboAmp Kit is ready for subsequent labeling and probing of cDNA microarrays. RNA amplification begins at the 3' end of the substrate molecule and therefore, amplified RNA product should not be used to prepare full-length cDNA libraries.

⚠ *Microarray experiments that compare labeled RNA amplified through linear amplification to labeled unamplified RNA should be designed to compare differential gene expression using RNA samples that are processed using identical methods.*

B. Performance Specifications

One round of amplification using a recommended input quantity of Control RNA supplied with the RiboAmp Kit typically yields about 30-50 µg of aRNA. The following table outlines recommended input amounts and subsequent outputs of aRNA for one or two rounds of amplification:

Recommended RNA inputs and typical aRNA outputs using the RiboAmp RNA Amplification Kit

Amplification Rounds	RNA Source	Minimum Input for Amplification (Total Cellular RNA)	Recommended Input (Total Cellular RNA)	Typical Output from Recommended Input (aRNA)
1	mRNA-rich Cells	500 ng	2-5 µg	30-50 µg
	mRNA-poor Cells	2 µg	5-10 µg	30-50 µg
2	mRNA-rich Cells	1 ng	1-20 ng	30-50 µg
	mRNA-poor Cells	4 ng	20-40 ng	30-50 µg
	LCM Samples	250 cells	500-2000 cells	~ 10-50 µg

Recommended inputs of high-quality RNA typically yield the output quantities shown. Output amounts were determined with Control RNA. Minimum input refers to the smallest quantity of high-quality RNA that will yield amplification product. Successful amplification is not guaranteed for minimum input quantities. The recommended and minimum number of LCM cells that may be used with the kit should serve as a general guide since RNA yield may vary based on cell types and other factors.

C. Quality Control

1. Functional Testing
Arcturus performs functional testing on each lot of kit materials using the protocol described in this manual. A one-round amplification is performed with 2 to 2.3 µg of Control RNA (supplied with the kit) with typical reaction yield of around 50 µg. A Certificate of Analysis is provided with each kit.

2. Reagent Testing
Arcturus tests each lot of enzymes to confirm activity. Buffer components must perform correctly under reaction or nucleic acid purification conditions.

3. Purification Column Testing
Purification columns are tested by lot to confirm the absence of nucleic acids and nuclease activity. Column nucleic acid binding and recovery performance must meet quality standards.

4. Visual Inspection
Finished kits are inspected for proper assembly.

D. Storage and Stability Information

Store the frozen reagent box (Box #1) at −20°C in a non frost-free freezer upon receipt. The Control RNA vial should be stored at −80°C for maximum stability.

Keep the room temperature box (Box #2) at room temperature. Kits stored under recommended conditions are stable for a minimum of six months from the date of delivery.

E. Material Safety and Data Sheets

Material Safety and Data Sheets (MSDS) for kit chemical components are available from Arcturus Technical Service or may be downloaded from www.arctur.com. Call 888.446.7911 or 650.962.3020, or send an email to techsupport@arctur.com.

F. Related Products from Arcturus

PicoPure™ RNA Isolation Kit
Cat. # KIT0202
For extraction and isolation of total RNA from small samples, particularly Laser Capture Microdissected (LCM) cells. Isolate RNA from as few as 10 cells in minimal volume. The PicoPure RNA Kit comes with optimized buffers, purification columns and an easy-to-use protocol to maximize recovery of high-quality total cellular RNA ready for amplification with the RiboAmp RNA Amplification Kit.

HistoGene™ LCM Frozen Section Staining Kit
Cat. # KIT0401
The HistoGene Kit is used to process tissue sections for LCM that maximizes the quality and yield of RNA from LCM cells. The Kit comes with all dehydration and staining reagents, disposable staining jars, specially treated slides, and a detailed protocol and troubleshooting guide.

PicoPure™ DNA Extraction Kit
Cat. # KIT0103
The PicoPure DNA Kit is optimized to maximize the recovery of genomic DNA from 10 or more cells captured by LCM. The kit comes with reagents and protocol tested to ensure complete extraction of DNA from LCM samples prepared with any standard tissue preparation procedure. DNA prepared using the kit is "PCR-ready" and needs no additional purification to perform amplification.

Cellex™ cDNA
Cellex cDNAs are prepared from high-quality mRNA obtained from Laser Capture Microdissected cells. The mRNA is then linearly amplified, using RiboAmp™ RNA Amplification, and converted to cDNA. From cell captures to PCR assays, quality is carefully controlled at every step to ensure Cellex cDNA represents the mRNA population of the captured cell type. Each Cellex cDNA includes enough for 20 quantitative or endpoint PCRs.

II. Kit Components

A. Reagents and Supplies in Kit

Box #1 - Frozen Reagent Box

Component	Vial Color	Vial #/Name
❑ 1st Strand Master Mix	Red	1
❑ 1st Strand Enzyme Mix	Red	2
❑ 1st Strand Nuclease Mix	Gold	-
❑ 2nd Strand Master Mix	White	1
❑ 2nd Strand Enzyme Mix	White	2
❑ IVT Buffer	Blue	1
❑ IVT Master Mix	Blue	2
❑ IVT Enzyme Mix	Blue	3
❑ DNase Mix	Blue	4
❑ Primer A	Beige	A
❑ Primer B	Pink	B
❑ Control RNA (0.2 µg/µL)	White	C
❑ Nuclease Free Water	White	W

⚠ *For maximum stability, store Control RNA at –80°C.*

Box #2 - Room Temperature Box

Component	Vial Color	Vial #/Name
❑ DNA Binding Buffer	Purple	DB
❑ DNA Wash Buffer	Purple	DW
❑ DNA Elution Buffer	Purple	DE
❑ RNA Binding Buffer	Green	RB
❑ RNA Wash Buffer	Green	RW
❑ RNA Elution Buffer	Green	RE
❑ Microcentrifuge Tubes	---	---
❑ DNA/RNA Purification Columns with collection tubes	---	---

III. Preliminary Notes

A. Recommendations for RNase-Free Technique

RNase contamination will cause experimental failure. Minimize RNase contamination by adhering to the following recommendations throughout your experiment:

- Wear disposable gloves and change them frequently.
- After putting on gloves, avoid touching surfaces that may introduce RNases onto the glove surface.
- Use only reagents supplied in the RiboAmp Kit. Substitutions of reagents or kit components may adversely affect yields or introduce RNases.
- Use only new, sterile RNase-free pipette tips and microcentrifuge tubes.
- Use only new plasticware that is nucleic acid-free.
- Work surfaces may be cleaned with commercially available RNase decontamination solutions prior to performing reactions.

B. Preparation/Isolation of RNA

RNA Quality

The success of amplification using the RiboAmp RNA Amplification Kit depends on the quality of the source RNA. Integrity is affected by exposure to internal and external sources of RNases. Avoiding RNA degradation due to intracellular RNases is often the most critical step in isolating good quality RNA. Isolation from cell cultures should be performed immediately after harvesting the cell to avoid RNase activity. Quiescent cells, such as those in tissue samples, require immediate freezing in embedding media to inactivate RNases. Subsequent processing of those samples requires methods that preserve RNA integrity.

Cells captured by LCM will yield high-quality RNA when appropriate protocols, such as those used in Arcturus' HistoGene LCM Frozen Section Staining Kit and PicoPure RNA Isolation Kit, are applied.

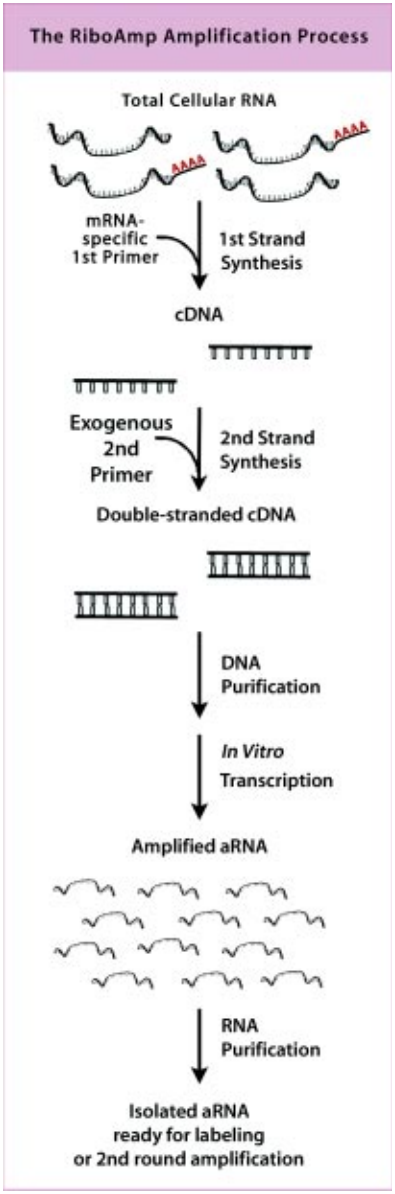
RNA Input

Using isolated total cellular RNA, rather than mRNA, for amplification is recommended to reduce the risk of high-abundance gene bias that may be introduced in mRNA purification. The RNA must be provided in RNase-free water, without the presence of organic solvents, salts, or contaminating cellular material. The RiboAmp Kit is optimized to amplify the mRNA fraction from low quantities of total cellular RNA. For recommended input quantities, refer to the Performance Specifications Table on page 4. When mRNA is used as input, adjust the input quantity to reflect the proper proportions in total cellular RNA. At low nucleic acid quantities, however, it may be necessary to add nucleic acid carrier to the sample prior to beginning the RiboAmp protocol. We recommended using RNA that has been treated with DNase to eliminate DNA contamination.

RNA Assessment

The quantity and quality of the input RNA may be assessed through the use of UV light absorbance quantitation. An A_{260} to A_{280} ratio of 1.7 to 2.1 indicates that RNA is of good quality for amplification. See Appendix A for details. UV spectrophotometry may not be possible with Laser Capture Microdissection samples.

RNA quality, especially when RNA is of extremely small quantity, may be assessed through quantitative, real-time PCR (Q-RT-PCR). See Appendix C. If starting quantity is sufficient, assessment using gel electrophoresis may be an option.



C. RNA Storage

RNA intended for use with the RiboAmp Kit should be used immediately after isolation or stored at –80°C until use. Avoid multiple freeze thaw cycles.

Amplified aRNA produced using the RiboAmp Kit should be used for labeling reactions as soon as possible. Alternatively, the aRNA may be stored at –80°C.

D. Additional Lab Equipment and Materials Required

1. Equipment

- ❑ Thermal Cycler with heated lid (highly recommended). If a thermal cycler is not available, you may use a combination of water baths, incubators, and heating blocks. Make sure each controlled-temperature component has reached the correct temperature and is ready for use before you begin. Do not allow incubation times and temperatures to deviate from the protocol.
- ❑ Microcentrifuge for 1.5 ml and 0.5 ml tubes (Eppendorf 5415D or similar)
- ❑ 0.5–10 µL pipettor
- ❑ 2–20 µL pipettor
- ❑ 20–200 µL pipettor
- ❑ 200–1000 µL pipettor
- ❑ Ice bath or cold block (4°C)
- ❑ Vortex mixer (optional)

2. Materials

- ❑ 0.5 mL or 0.2 mL RNAse-free microcentrifuge tubes
- ❑ Nuclease-free pipette tips

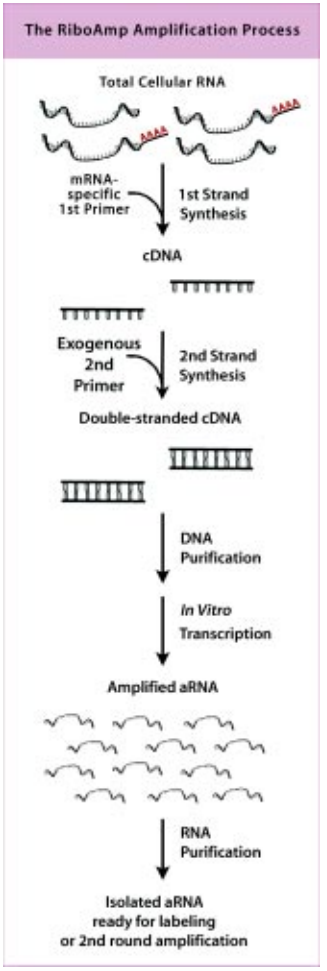
3. Reagents

- ❑ Nucleic Acid Carrier (Recommended if performing two rounds of amplification with RNA input isolated by methods other than the use of the PicoPure RNA Isolation Kit) such as: Poly (I) – Polyinosinic acid OR Poly (dI-dC) – Poly-deoxy-inosinic-deoxy-cytidylic acid

IV. Protocol

A. Overview

The RiboAmp RNA Amplification Kit amplifies nanogram or low-microgram amounts of starting RNA. The RiboAmp Kit utilizes a five-step process for linear amplification of the mRNA fraction of total cellular RNA: (a) A first-strand synthesis reaction yields cDNA incorporating a T7 promoter. (b) A second-strand synthesis reaction utilizing exogenous primers yields double-stranded cDNA. (c) A specially designed purification column purifies the double-stranded cDNA. (d) Following cDNA isolation, *in vitro* transcription (IVT) utilizing T7 RNA polymerase yields antisense RNA (aRNA) which is then (e) isolated with the purification column.



One round of amplification can be completed in less than one day, while two rounds of amplification can be completed through the start of the second round IVT in one full day and completed the following morning in one hour. The protocol enables one round of amplification, aRNA labeling, and set up of microarray hybridization in one working day.

The use of exogenous primers in the 2nd strand synthesis maximizes the reliability of the reaction and minimizes the reaction times required. This approach produces amplified aRNA with greater uniformity in length. As a result, labeling reactions in preparation for microarray hybridization produce labeled cDNA with highly uniform fluorescent dye incorporation. In other words, uniform incorporation of fluorescent molecules per unit of RNA transcript is achieved when starting with aRNA of uniform length. Microarray hybridization and subsequent fluorescent intensity measurement is likely to be more indicative of the actual expressed gene copy number.

The proprietary amplification method used in this kit produces aRNA in microgram quantities for subsequent labeling and hybridization onto expression microarrays. The bulk of the aRNA product from one round of amplification is generally 250–1800 bases in length; aRNA product ranges from below 200 to over 600 bases following a second round of amplification. As a result, direct comparison of microarray data from studies using

unamplified, full-length RNA and amplified aRNA prepared using the RiboAmp Kit may not be possible. However, numerous studies using expression microarrays and amplified aRNA as probe have revealed differential expression patterns verified by other methods.

The RiboAmp Kit produces unlabeled aRNA that can be subsequently used for reverse transcription-based incorporation of labeled nucleotides to produce labeled cDNA (sense strand). For microarrays requiring labeled aRNA (such as GeneChip® Probe Arrays), protocols for incorporation of label during the IVT reaction can be implemented (see Appendix F).

B. Time Requirements

The table below presents typical time requirements for completion of the RiboAmp Kit protocol. Times reflect total handling and reaction times of each step. Note that there are stopping points for pausing the amplification process, and the times presented reflect a continuous, uninterrupted process.

Steps	1st Round	2nd Round
1st Strand Synthesis	1.0 hours	1.0 hours
2nd Strand Synthesis	0.5 hours	0.5 hours
cDNA Purification	0.5 hours	0.5 hours
In Vitro Transcription	3.0 hours	4.5 hours
aRNA Purification	0.5 hours	0.5 hours
Total	5.5 hours	2.0 hours (pre-IVT) 7.0 hours (total)

C. Thermal Cycler Programming

A thermal cycler provides a convenient method of incubating reaction samples according to the specified temperatures and times in the RiboAmp Kit protocol. A thermal cycler program for use with the RiboAmp Kit appears on the next page. The program is not intended for automatic progression from one time and temperature set to another. The program lists a 4°C hold after each incubation or incubation cycle when it is necessary to remove the reaction sample from the thermal cycler to add reagents. After addition of reagents, place the reaction sample back into the thermal cycler and resume the program.

Using a thermal cycler with a heated lid is important. The heated lid ensures proper temperature distribution within the reaction tube and prevents evaporative condensation that alters the reaction mixture concentrations.

Program

Round One		
°C		
65	5 minutes	
4	hold	
42	45 minutes	*1st Strand Synthesis*
4	hold	
37	20 minutes	
95	5 minutes	
4	hold	
95	2 minutes	
4	hold	
25	5 minutes	*2nd Strand Synthesis*
37	10 minutes	
70	5 minutes	
4	hold	
42	3 hours (optional: 4 hours)	
4	hold (optional overnight hold)	*IVT*
37	15 minutes	
4	hold	

Round Two		
°C		
65	5 minutes	
4	hold	
25	10 minutes	*1st Strand Synthesis*
37	45 minutes	
4	hold	
95	2 minutes	
4	hold	
37	15 minutes	*2nd Strand Synthesis*
70	5 minutes	
4	hold	
42	4-6 hours	
4	hold (optional overnight hold)	*IVT*
37	15 minutes	
4	hold	

⚠ *The 4°C steps in the thermal cycler program allow for buffer and reagent addition and mixing steps at certain points during the amplification process and are not intended for indefinite hold unless noted.*

D. Detailed Protocol

1. Protocol Notes

a. When adding reagent to samples or master mixes, pipette mixtures up and down several times to ensure complete transfer of reagent from the pipette tip.

b. Prior to the first use of an enzyme, briefly microcentrifuge the vial to ensure that all enzyme is collected at the bottom of the vial. Enzyme may collect on the vial wall or cap during shipment.

c. Prior to each incubation, mix samples thoroughly to ensure process performance.

d. If you choose to use a vortex to mix your reactions, USE ONLY THE LOWEST VORTEX SETTING and vortex for three seconds or less. Excessive mixing can lead to poor amplification results.

e. Use a microcentrifuge to spin down all components and samples following each mixing step.

f. Clean all amplification process equipment with an RNase eliminator such as RNase AWAY® (Molecular Bio Products) to minimize the risk of RNase contamination.

2. Prepare Sample and Reagents

a. Thaw frozen components and mix with gentle vortexing or by inverting the tubes several times, and place on ice. When enzyme mixtures must be removed from –20°C storage for use, always keep them in a cold block or in an ice bucket at the lab bench.

b. Allow *In Vitro* Transcription (IVT) reagents (Blue-labeled Vials) to assume room temperature (22–25°C). Dissolve all visible solids and mix prior to use.

c. The RiboAmp RNA Amplification Kit is optimized for the input of total cellular RNA For input of mRNA, total yield may be less than 1000-fold amplification due to substrate limitations unless lower input quantities are used.

d. Resuspend RNA in nuclease-free water prior to amplification. Avoid using organic solvents in RNA isolation procedures. Trace amounts of organic solvents that carry

over into amplification reactions will impair the RiboAmp Kit amplification process.

e. Isolate RNA from LCM samples using the PicoPure RNA Isolation Kit (KIT0202) for best results. RNA isolated by the PicoPure Kit is ready to use in the RiboAmp Kit without further processing. Perform the optional DNase treatment protocol prior to eluting.

f. RNA should be DNase treated prior to input into the RiboAmp Kit to eliminate possible genomic DNA interference of the amplification process. DNase enzyme and buffers must be removed prior to RNA input into the RiboAmp Kit protocol. DNase treatment may be incorporated directly into the protocol of the PicoPure RNA Isolation Kit or other available purification column-based approaches.

3. Special Notes for Performing Two Rounds of Amplification

a. We recommend isolating RNA using the PicoPure RNA Isolation Kit if working with LCM samples. If you use a different method to isolate RNA (or if working with non-LCM samples), AND if the starting input quantity of RNA into the RiboAmp Kit is less than 100 ng, add a nucleic acid carrier such as Poly (I) or Poly (dI-dC) (Amersham, Sigma, Roche, etc…) to the input RNA to prevent non-specific RNA priming. Add 200 ng of the carrier to the input RNA prior to beginning 1st Strand Synthesis of Round One. The final volume of the sample should be 10 µL. It is not necessary to add carrier if RNA isolation was performed using the PicoPure RNA Isolation Kit.

b. When performing two rounds of amplification starting from low nanogram quantities of RNA, amplify 2.0 µg Control RNA (supplied with the kit) with the first round of amplification to assess the quality of the amplification process. First-round amplified RNA from low starting quantities will often not be detectable. Inspect your Control RNA one-round amplification results by UV spectrophotometry and gel electrophoresis to ensure successful amplification before committing test samples to a second round of amplification.

4. Protocol for Performing One Round of Amplification

Round One: 1st Strand Synthesis

1. Prepare RNA sample in a total volume of 10.0 µL nuclease-free water and place in a 0.5 mL or 0.2 mL RNase-free microcentrifuge tube. Refer to the specifications table on page 4 for information regarding starting RNA quantities.
2. Add 1.0 µL of Primer A (Beige-labeled Vial - A), mix well and spin down.
3. Incubate at 65°C for five minutes then chill the samples to 4°C for at least one minute. Hold the sample at 4°C until ready to proceed. Spin the contents down before proceeding to the next step.
4. Thaw 1st Strand Synthesis components (Red-labeled Vials) and place on ice (1st Strand Enzyme Mix does not require thawing and can be placed directly on ice.) You may add reagents directly to the sample. If you are performing several amplifications, you will find it more convenient to prepare Complete 1st Strand Synthesis Mix according to the following table.

Complete 1st Strand Synthesis Mix

Component	Amount	Red Vial #
1st Strand Master Mix	7 µL	1
1st Strand Enzyme Mix	2 µL	2
Total per sample	9 µL	

5. To the chilled sample, add 9.0 µL Complete 1st Strand Synthesis Mix. Mix thoroughly, spin down, and incubate at 42°C for 45 minutes.
6. Chill the sample to 4°C for at least one minute, then spin down briefly.
7. (Optional) You may remove a 2.0 µL sample at this point in the protocol to assess the integrity of the starting mRNA by Q-RT-PCR. See Appendix C.

Beige

If you intend to perform 2 rounds of amplification, see Special Notes for Performing Two Rounds of Amplification in Chapter IV, Section D, 3.

Primer A must be thawed, thoroughly mixed, and spun down prior to use.

1st Strand Synthesis reagents must be thawed, thoroughly mixed with all solids dissolved, and maintained at 4°C until used.

Red

Red

8. Add 2.0 µL of 1st Strand Nuclease Mix (Gold-labeled Vial) to the sample, mix thoroughly, spin down, and incubate at 37°C for 20 minutes.
9. Incubate the sample at 95°C for five minutes.
10. Chill the sample to 4°C for at least one minute and hold at that temperature until ready to proceed. Spin down briefly.

OK *It is okay to stop at this point in the protocol. Sample may be stored at –20°C overnight.*

Gold

⚠ *1st Strand Nuclease Mix must be brought to 4°C, thoroughly mixed, and spun down prior to dispensing.*

Round One: 2nd Strand Synthesis

1. Add 1.0 µL of Primer B (Pink -labeled Vial - B) at 4°C. Mix thoroughly and spin down.
2. Incubate sample at 95°C for two minutes, then chill and maintain the sample at 4°C for at least two minutes.
3. Thaw 2nd Strand Synthesis components (White-labeled Vials) and place on ice. 2nd Strand Enzyme Mix does not require thawing and can be placed directly on ice. Reagents may be added directly to the sample, or, if performing several amplifications, a Complete 2nd Strand Synthesis Mix may be prepared according to the following table:

Complete 2nd Strand Synthesis Mix

Component	Amount	White Vial #
2nd Strand Master Mix	29 µL	1
2nd Strand Enzyme Mix	1 µL	2
Total per sample (Store at 4°C until use.)	30 µL	

4. Add 30 µL of the Complete 2nd Strand Synthesis Mix to the sample. Mix thoroughly and spin down.
5. Incubate the sample as follows:
   - 25°C 5 minutes
   - 37°C 10 minutes
   - 70°C 5 minutes
   - 4°C Hold until ready to proceed (up to a maximum of 30 minutes).

Pink

Primer B must be thawed, thoroughly mixed, and spun down prior to use.

2nd Strand Synthesis reagents must be thoroughly mixed with all solids dissolved, and maintained at 4°C until used.

White

White

Round One: cDNA Purification

1. Add 250 µL of DNA Binding Buffer (DB) to a DNA / RNA Purification Column seated in the collection tube provided. Hold for five minutes at room temperature. Centrifuge at 16,000 x *g* for one minute.
2. Add 200 µL of DNA Binding Buffer (DB) to the 2nd Strand Synthesis sample tube, mix well, and pipette the entire volume into the purification column.
3. To bind cDNA to column, centrifuge at 100 x *g* for two minutes (or lowest speed setting available), immediately followed by a centrifugation at 10,000 x *g* for 30 seconds to remove flowthrough.
4. Add 250 µL of DNA Wash Buffer (DW) to the column and centrifuge at 16,000 x *g* for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x *g* for one minute.
5. Discard the flowthrough and collection tube.
6. Place the column into the provided 0.5 mL microcentrifuge tube and carefully add 16 µL of DNA Elution Buffer (DE) onto the center of the purification column membrane. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of DE into the membrane). Gently tap the purification column to distribute the buffer, if necessary. Incubate for one minute at room temperature.

Purple

⚠ *DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed by shaking before use. A precipitate may form during long term storage. Dissolve precipitate prior to use by mixing. If necessary, warm the DB vial to redissolve.*

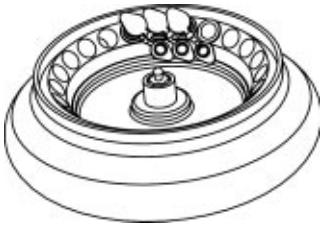
Purple

⚠ *Avoid splashing flowthrough in the collection tube onto the column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge the column at16,000 x g to remove liquid.*

Purple

7. Place the assembly into the centrifuge as shown and centrifuge at 1,000 x *g* for one minute, followed immediately by 16,000 x *g* for one minute. Discard the column and retain the elution containing the cDNA in the micro-centrifuge tube for further processing.

OK *It is safe to stop at this point in the protocol. You may store the sample overnight at –20°C.*



To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as ilustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.

Round One: *In Vitro* Transcription (IVT)

1. Thaw all IVT Reaction components on ice (IVT Enzyme Mix does not require thawing and can be put directly on ice. Bring components to room temperature just prior to use. Place components back onto ice or refreeze immediately after dispensing the reagent. Do not leave reagents at room temperature for any extended period of time. Reagents may be added directly to the sample. If you are performing several amplifications, a Complete IVT Reaction Mix may be prepared according to the following table:

IVT reaction components must be thawed thoroughly mixed with all solids dissolved, and brought to room temperature just before use.

Complete IVT Reaction Mix

Component	Amount	Blue Vial #
IVT Buffer	8 µL	1
IVT Master Mix	12 µL	2
IVT Enzyme Mix	4 µL	3
Total per sample	24µL	

Blue

Blue

Blue

Components must be added and mixed in the order listed in the table. Mix the Complete IVT Reaction Mix gently by brief vortexing at the lowest setting, or by flicking the tube after addition of each component.

2. Add 24 µL of the Complete IVT Reaction Mix to each sample, mix thoroughly, spin down, and incubate at 42°C for 3 hours. (Optional: four hour incubation may be used for additional aRNA yield) Cool samples to 4°C.

OK *At this point in the protocol, you may hold the reaction mixture at 4°C in the thermal cycler overnight.*

3. Add 2 µL DNase Mix (Blue-labeled Vial #4). Mix thoroughly and spin down. Incubate at 37°C for 15 minutes. Cool the sample(s) to 4°C. Continue on to the purification of aRNA immediately.

Blue

DNase Mix must be thoroughly mixed and held at 4°C until used.

RNA may be adversely affected if not purified immediately after DNase treatment.

Round One: Antisense RNA (aRNA) Purification

1. Add 250 µL of RNA Binding Buffer (RB) to a new DNA/RNA Purification Column and hold for five minutes at room temperature. Centrifuge at 16,000 x *g* for one minute.
2. Add 200 µL of RNA Binding Buffer (RB) to the IVT Reaction sample and mix thoroughly. Pipette the entire sample volume into the purification column.
3. To bind aRNA to the column, centrifuge at 100 x *g* for two minutes (or lowest speed setting available), immediately followed by a centrifugation at 10,000 x *g* for 30 seconds to remove flowthrough.
4. Add 200 µL of RNA Wash Buffer (RW) to the purification column and centrifuge at 10,000 x *g* for one minute.
5. Add 200 µL of fresh RNA Wash Buffer (RW) to the purification column, and centrifuge at 16,000 x *g* for two minutes. Check the purification column for any residual wash buffer. If any wash buffer reamins, re-centrifuge at 16,000 x *g* for one minute.
6. Discard the flowthrough and used collection tube.
7. Place the purification column into a new 0.5 mL microcentrifuge tube provided in the kit and carefully add of RNA Elution Buffer (RE) directly onto the center of the purification column membrane, 30 µL if stopping with one round or 11 µL if going on to a second round. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absoption of RE into the membrane). Gently tap the purification column to distribute the buffer, if necessary. Incubate at room temperature for 1 minute.

Green

⚠ *RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during longterm storage. Dissolve precipitate prior to use by mixing. If necessary, warm the RB vial to re-dissolve.*

Green

⚠ *Avoid splashing flowthrough in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge the column at 16,000 x g to remove the liquid.*

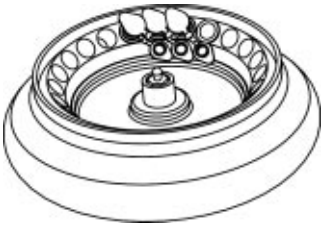
Green

8. Place the assembly into the centrifuge as shown, and centrifuge at 1,000 x *g* for one minute, immediately followed by 16,000 x *g* for one minute. Discard the purification column and retain the elution containing the aRNA in the microcentrifuge tube for further processing.

9. Measure the O.D. of the product at A_{260} and A_{280} (Refer to Appendix B). To achieve an appropriate dilution for measurement, remove a 1–5 µL aliquot and dilute with distilled water to 100 µL. If the aRNA concentration meets your experimental requirements, you may stop at this point; otherwise proceed to Step 11.

10. Perform electrophoretic analysis to confirm the presence of amplification product unless you intend to do a second round of amplification. The aRNA will appear as a smear on the gel from 250 to 1800 bases in length, with the majority of product around 600 bases in length. Starting inputs of less than 100 ng of total RNA may not generate sufficient aRNA to be measured spectrophotometrically or visualized on a gel.

The purified aRNA is ready for use in a labeling reaction and for use in a second round of amplification as described in the following section of this User Guide. Purified aRNA may be stored at –70°C.



⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as ilustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

End of Round One

5. Protocol for Performing Second Round of Amplification

A second round of amplification can be performed to increase yield of the amplification, if necessary. In second round amplification, purified aRNA product from Round One is used to produce double-stranded cDNA, which in turn is used as template for an *in vitro* transcription reaction.

There are two significant differences between the first-round and second-round amplification protocols. 1) Since Primer A is a component of 2nd Strand Synthesis, and Primer B is a component of 1st Strand Synthesis, reaction temperatures and incubation intervals are different. 2) The second-round amplification protocol does not make use of 1st Strand Nuclease Mix. Do not use the following second-round amplification protocol without first performing a first round of amplification.

The aRNA product produced after the second round of amplification is somewhat shorter than that formed from one round. Typically, the bulk of the aRNA visualized through gel electrophoresis will range from under 200 to over 600 bases.

Round Two: 1st Strand Synthesis

1. Prepare aRNA sample(s) from Round One .
2. Add 1.0 µL of Primer B (Pink-labeled Vial - B), mix well and spin down.
3. Incubate at 65°C for five minutes then chill the sample(s) to 4°C for at least one minute. Hold the sample at 4°C until ready to proceed. Spin the contents down before proceeding to the next step.
4. Thaw 1st Strand Synthesis components (Red-labeled vials) and place on ice (1st Strand Enzyme Mix does not require thawing and can be placed directly on ice. Reagents may be added directly to the sample, or, if you are performing several amplifications, a Complete 1st Strand Synthesis Mix may be prepared according to the following table:

Complete 1st Strand Synthesis Mix

Component	Amount	Red Vial #
1st Strand Master Mix	7 µL	1
1st Strand Enzyme Mix	2 µL	2
Total per sample	9 µL	

5. To the chilled sample, add 9.0 µL Complete 1st Strand Synthesis Mix. Mix thoroughly, spin down, and incubate the reaction at 25°C for 10 minutes then 37°C for 45 minutes.
6. Chill the sample to 4°C for at least one minute, but less than 30 minutes, then spin down briefly.

OK *It is okay to stop at this point in the protocol. Sample(s) may be stored overnight at –20°C.*

Pink

⚠ *Primer B must be thawed, thoroughly mixed, and spun down prior to use.*

⚠ *1st Strand Synthesis reagents must be thawed, thoroughly mixed with all solids dissolved and maintained at 4°C until used.*

Red

Red

Round Two: 2nd Strand Synthesis

1. Add 1.0 µL of Primer A (Beige-labeled Vial - A) to the sample at 4°C. Mix thoroughly and spin down.
2. Heat sample at 95°C for two minutes. Cool sample to 4°C for at least two minutes.
3. Thaw 2nd Strand Synthesis components (White-labeled Vials) and place on ice. 2nd Strand Enzyme Mix does not require thawing and can be placed directly on ice. Reagents may be added directly to the sample, or, if you are performing several amplifications, a Complete 2nd Strand Synthesis Mix may be prepared according to the following table:

Complete 2nd Strand Synthesis Mix

Component	Amount	White Vial #
2nd Strand Master Mix	29 µL	1
2nd Strand Enzyme Mix	1 µL	2
Total per sample (Store at 4°C until use.)	30 µL	

4. Add 30 µL of the Complete 2nd Strand Synthesis Mix to the sample, mix thoroughly, and spin down.
5. Incubate the sample as follows:
   - 37°C 15 minutes
   - 70°C 5 minutes
   - 4°C Hold until ready to proceed to a maximum of 30 minutes).

Beige

Primer A must be thawed, thoroughly mixed, and spun down prior to use.

2nd Strand Synthesis reagents must be thawed, thoroughly mixed, with all solids dissolved, and maintained at 4°C until used.

White

White

Round Two: cDNA Purification

1. Add 250 µL of DNA Binding Buffer (DB) to a DNA / RNA Purification Column seated in the collection tube provided. Hold for five minutes at room temperature. Centrifuge at 16,000 x *g* for one minute.
2. Add 200 µL of DNA Binding Buffer (DB) to the 2nd Strand Synthesis sample tube, mix well, and pipette the entire sample volume into the purification column.
3. To bind the cDNA to the column, centrifuge at 100 x *g* for two minutes (or lowest speed setting available), immediately followed by a centrifugation at 10,000 x *g* for 30 seconds to remove flowthrough.
4. Add 250 µL of DNA Wash Buffer (DW) to the purification column and centrifuge at 16,000 x *g* for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x *g* for one minute.
5. Discard the flowthrough and collection tube.
6. Place the column into the provided 0.5 mL microcentrifuge tube and carefully add 16 µL of DNA Elution Buffer (DE) directly onto the center of the purification column membrane. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of DE into membrane). Gently tap the purification column to distribute the buffer, if necessary. Incubate for one minute at room temperature.

Purple

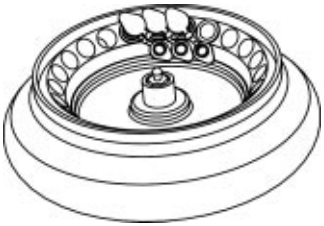
DNA Binding Buffer (DB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the DB vial to re-dissolve.

Purple

Avoid splashing flowthrough in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the purification column, recentrifuge at 16,000 x g to remove liquid.

Purple

7. Place the assembly into the centrifuge as shown, and centrifuge at 1,000 x *g* for one minute and then at 16,000 x *g* for one minute Discard the column and retain the elution containing the cDNA in the microcentrifuge tube for further processing.



OK *It is okay to stop at this point in the protocol. Sample may be stored overnight at –20°C.*

⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as ilustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

Round Two: *In Vitro* Transcription

1. Thaw all IVT Reaction components on ice (IVT Enzyme Mix does not require thawing and can be put directly on ice. Bring components to room temperature just prior to use. Place components back onto ice or re-freeze immediately after dispensing the reagent. Do not leave the reagent at room temperature for any extended period of time. Reagents may be added directly to the sample, or, if you are performing several amplifications, a Complete IVT Reaction Mix may be prepared according to the following table:

IVT reaction components must be thawed, thoroughly mixed with all solids dissolved, and allowed to warm to room temperature just before use.

Complete IVT Reaction Mix

Component	Amount	Blue Vial #
IVT Buffer	8 µL	1
IVT Master Mix	12 µL	2
IVT Enzyme Mix	4 µL	3
Total per sample	24 µL	

Blue

Blue

Blue

Components must be added and mixed in the order listed in the table. Mix the Complete IVT Reaction Mix gently by brief vortexing at the lowest setting, or by flicking the tube after addition of each component.

DNase Mix must be thoroughly mixed and held at 4°C until used.

2. Add 24 µL of the Complete IVT Reaction Mix to each sample, mix thoroughly, spin down, and incubate at 42°C for four hours. (Optional: the IVT reaction may be incubated for up to six hours if additional yield is desired) Chill sample(s) to 4°C.

OK *At this point in the protocol, you may hold the reaction mixture at 4°C in the thermal cycler overnight.*

3. Add 2 µL DNase Mix (Blue-labeled Vial #4), mix thoroughly, and spin down. Incubate at 37°C for 15 minutes. Cool the sample(s) to 4°C. Continue on to aRNA Purification immediately.

Blue

DNase Mix must be thoroughly mixed and held at 4°C until used.

RNA may be adversely affected if not purified immediately after DNase treatment.

Round Two: Antisense RNA (aRNA) Purification

1. Add 250 µL of RNA Binding Buffer (RB) to a new DNA/RNA Purification Column and hold for five minutes at room temperature. Centrifuge at 16,000 x *g* for one minute.
2. Add 200 µL of RNA Binding Buffer (RB) to the IVT Reaction sample and mix thoroughly. Pipette the entire sample volume into the purification column.
3. Centrifuge at 100 x *g* for two minutes (or lowest speed setting available), immediately followed by a centrifugation at 10,000 x *g* for 30 seconds.
4. Add 200 µL of RNA Wash Buffer (RW) to the purification column and centrifuge at 10,000 x *g* for one minute.
5. Add 200 µL of fresh RNA Wash Buffer (RW) to the column, and centrifuge at 16,000 x *g* for two minutes. Check the purification column for any residual wash buffer. If any wash buffer remains, re-centrifuge at 16,000 x *g* for one minute.
6. Discard the flowthrough and used collection tube.
7. Place the purification column into a new 0.5 mL microcentrifuge tube provided in the kit and carefully add 30 µL of RNA Elution Buffer (RE) directly onto the center of the purification column membrane. (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of RE into the membrane). Gently tap the purification column to distribute the buffer, if necessary. Incubate at room temperature for 1 minute.

Green

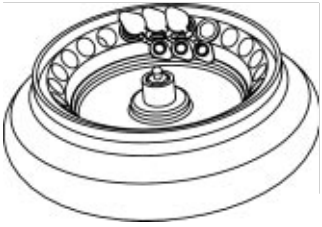
RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the RB vial to redissolve.

Green

Avoid splashing flowthrough in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the column, re-centrifuge the column at 16,000 x g to remove the liquid.

Green

8. Place the assembly into the centrifuge as shown, and centrifuge at 1,000 x *g* for one minute and then at 16,000 x *g* for one minute. Discard the column and retain the elution containing aRNA in the microcentrifuge tube for further processing.
9. Measure the O.D. of the product at A_{260} and A_{280} (Refer to Appendix A). To achieve an appropriate dilution for measurement, remove a 1–5 µL aliquot and dilute with distilled water to 100 µL. Perform electrophoretic analysis to confirm the presence of amplification product. The aRNA will appear as a smear on the gel ranging from under 200 to over 600 bases in length.



⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as ilustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

The purified aRNA is ready for use in a labeling reaction. Purified aRNA may be stored at –70°C.

⚠ *Do not allow sample(s) to dry completely! Quantitative recovery of material will be severely compromised.*

End of Round Two

V. Troubleshooting

A. Amplification Yield is Poor

Cause: Starting RNA sample quality varies.

Suggestion: If you observe low yields with different RNA samples, run an amplification using the Control RNA provided in the RiboAmp Kit to verify kit functionality.

Cause: Starting RNA sample quality has been compromised.

Suggestion: The greatest factor affecting amplification efficiency is the integrity of the RNA used in the RiboAmp amplification process. Suspend RNA in nuclease-free water prior to amplification. Avoid using organic solvents such as phenol in RNA isolation procedures. Trace amounts of organic solvents that carry over into amplification reactions will impair the amplification process. If input RNA is from cells obtained by LCM, use specialized LCM sample preparation protocols designed to preserve RNA. HistoGene and PicoPure Kits from Arcturus are optimized to preserve the integrity of RNA and maximize recovery. Quantitative real-time PCR of 1st Strand Synthesis product can be used to verify the quality and quantity of the RNA input.

Cause: Reagent concentrations in reaction mixtures are incorrect due to inadequate thawing or mixing.

Suggestion: Ensure all reagents are completely thawed, mixed, and all solids are dissolved prior to use.

Cause: Reagent concentrations in the reaction mixtures are incorrect due to inadequate reaction volume collection in the reaction tube.

Suggestion: Thoroughly thaw and mix all reagents prior to dispensing. Ensure all reagents are dispensed at proper volumes. Briefly spin down the reaction mix prior to incubation to ensure all reagents are collected in the reaction volume and the reaction mix has the proper concentrations of reagents.

Cause: Reagent concentrations in reaction mixtures are incorrect due to evaporative condensation onto the wall of the reaction tube during incubation.

Suggestion: Briefly spin down sample, following incubation steps to maintain proper volumes and concentrations of reagents and ensure that all nucleic acid templates are mixed with reaction components.

Cause: Incubation temperatures are incorrect.

Suggestion: Verify accuracy of all incubation temperatures. If you are using a thermal cycler, make sure that the programmed temperatures read correctly and the instrument has been calibrated to establish and maintain accurate temperature settings.

Cause: RNA yield is diminished during column purification.

Suggestion: Verify centrifugal force used during nucleic acid purification. Improper binding, washing, and elution centrifugal forces can decrease the recovery of nucleic acid from the purification column. Microcentrifuges should be calibrated to deliver the correct centrifugal force.

Cause: The RNA sample was vacuum centrifuged to dryness.

Suggestion: Monitor RNA concentration to prevent concentration to dryness. RNA is difficult to resuspend after drying, and loss of RNA may occur. Check the progress of the concentration periodically if the conditions have not been thoroughly optimized.

B. Low Molecular Weight Product Appears on Gel

Occasionally, a predominant band below the expected aRNA smear will appear on a gel. This band will lead to improper estimation of yield and may result in high backgrounds on microarrays. The RiboAmp Kit components are formulated and tested to avoid the synthesis of this material. However, if low molecular weight material is present, one of the following may be occurring:

Cause: Quality of the starting RNA is inadequate.

Suggestion: Poor RNA quality can lead to the formation of the reaction artifact, visible as a low molecular weight band. Check the quality of your input RNA. One approach is to utilize the Agilent Lab-on-a-Chip System with a RNA LabChip® kit. For additional recommendations to check for the quality of input RNA, contact Arcturus Technical Support.

Cause: Quantity of starting RNA is inadequate.

Suggestion: Verify the quantity of starting RNA. The absence of RNA or extremely low amounts of starting RNA may lead to formation of low molecular weight product. When working with low quantities, include nucleic acid carrier such as Poly I in the RNA input.

Cause: Concentrations of Primer A, Primer B, or 1st Strand Nuclease Mix are incorrect due to inadequate thawing or dispensing.

Suggestion: Thaw and thoroughly mix each reagent vial prior to dispensing. If incompletely thawed and mixed, the concentrations of these reagents may not be dispensed at optimal concentrations for the reaction. Ensure that all pipettes are properly calibrated to dispense correct volumes.

Cause: Concentrations of Primer A, Primer B, or 1st Strand Nuclease Mix are incorrect due to inadequate mixing or reaction volume collection inside the reaction tube.

Suggestion: Thoroughly mix and spin down the sample after adding Primer A, Primer B, or 1st Strand Nuclease Mix into the reaction mix and prior to incubation. This ensures the correct concentration of primers or nuclease mix in each respective reaction mix.

C. Haze of High Molecular Weight Product Appears on Gel

Cause: Quality or quantity of starting RNA is inadequate.

Suggestion: Absence or extremely low amounts of quality starting RNA may lead to formation of high molecular weight haze visible on an agarose gel. Ensure that the quality of the starting material is good by methods such as using the Agilent Lab-on-a-Chip System with a RNA LabChip kit. Determine that the concentration of the RNA is correct before starting amplification.

Cause: Concentration of Primer A and/or Primer B are incorrect due to reagent volume adhesion to the inside of the reaction tube.

Suggestion: To ensure correct primer concentrations, thoroughly mix and spin down the sample after adding Primer A or Primer B into the reaction mix and prior to incubation.

VI. Appendices

A. RNA Yield and Purity Determination

RNA quantitation using ultraviolet light absorbance quantitation of RNA is the simplest approach to determining amplification yield. An absorbance reading at 260 nm (A_{260}) using a spectrophotometer is taken on a diluted aliquot of aRNA. Typically, a 1:25 to 1:50 dilution of aRNA in nuclease free water is sufficient.

For single-stranded RNA, a measurement of A_{260} = 1.0 corresponds to 40 µg/mL. The yield can by calculated by:

$$(A_{260})\ (\text{dilution factor})\ (40) = \mu g/mL\ \text{RNA}$$

Measuring A_{280} and calculating the A_{260}/A_{280} ratio will indicate the purity of the RNA sample. An A_{260}/A_{280} ratio that approaches 2.0 indicates very pure RNA.

B. Amplified aRNA Labeling

Labeling reactions through reverse transcription incorporation of labeled nucleotides may be primed with degenerate primers or random hexamer primers. Note that aRNA is truncated and does not incorporate the same amount of dye as full-length RNA transcripts.

Protocols for labeling are being developed and validated by Arcturus. For availability of protocols, please contact Arcturus Technical Support at techsupport@arctur.com or visit our web site at www.arctur.com. See Appendix F if you are using the RiboAmp Kit with GeneChip® Probe Arrays (Affymetrix).

C. Assessment of RNA Sample Quality

Although gel electrophoresis is a common approach to assessing RNA quality, it is often not possible to run a gel on the small quantities of input RNA used for amplification with the RiboAmp Kit. Therefore, you may wish to assess the input RNA quality after 1st Strand Synthesis using quantitative real time PCR (Q-RT-PCR). You may use the following guidelines:

Use RNase-free technique. Wipe all surfaces and equipment with RNase decontamination solution, use RNase-free solutions and plasticware, and wear disposable gloves.

1. Perform amplification following the RiboAmp Kit protocol.
2. During Round One 1st Strand Synthesis, remove 2 µL of the sample as explained in the optional step # 7 on page 16.
3. Pipette into a new 0.5 mL microcentrifuge tube .
4. Add 8 µL of nuclease-free water. This is the diluted cDNA template.
5. Mix the sample well. Spin down and store on ice until ready to use.
6. Proceed according to protocols and Instruction Manuals for the Q-RT-PCR system utilized. Use 2 µL of diluted cDNA template (from step 4).

Refer to the Q-RT-PCR system manual for details concerning interpretation of data.

D. Assessment of aRNA Quality Using the Agilent Lab-on-a-Chip System

The Agilent Lab-on-a-Chip System provides a fast and effective approach to assessing the quality of RNA samples. The system requires very small quantity of sample. Refer to the Agilent 2100 bioanalyzer and RNA LabChip Kit Instruction Manuals for details.

Equipment and Materials Required

Agilent 2100 bioanalyzer System (Agilent)
RNA 6000 Nano Assay Kit (Agilent)
Ice or cold block (4°C)
Spectrophotometer

Before you begin, refer to the instruction manual for the RNA 6000 Nano Assay Kit. Prepare necessary reagents and supplies as required by the kit.

Use RNase-free technique. Wipe all surfaces and equipment with RNase decontamination solution, use RNase-free solutions and plasticware, and wear disposable gloves.

Protocol

1. Determine the concentration of the aRNA generated through RiboAmp by UV spectrophotometry. (Refer to Appendix A.)
2. Based on the optical density reading, prepare a dilution of the sample to a concentration of 200–300 ng/µL.
3. Store the sample on ice or in a cold block until ready to load on to the RNA chip.
4. Follow the RNA 6000 Nano Assay Kit protocol, loading 1 µL of the diluted sample (from step 2).

For details of data interpretation, refer to the bioanalyzer instrument manual. Good quality aRNA appears on the bioanalyzer as a single broad peak. The bulk of the RNA ranges in size from 200 to 800 bases long.

E. Analysis of aRNA by Agarose Gel Electrophoresis

Analysis of aRNA using agarose gel electrophoresis is one method to visualize the aRNA profile and relative quantity after amplification. Standard protocols for agarose gel electrophoresis can be used. The following is a suggested protocol using commercially available reagents.

Materials

1.25% Agarose Portrait Gel or 1.25 Agarose Medium Gel (EmbiTec cat. # GE-6010 or GE-6030)
10X RNA MOPS Running Buffer (EmbiTec cat. # EC-1020)
2X Gel Loading Buffer (various)
RNA Ladder (various)
SYBR® Gold Nucleic Acid Gel Stain (Molecular Probes cat. # S-11494) or Ethidium Bromide Stain
Nuclease-free Water

Protocol

1. Determine the concentration of the aRNA by UV absorbance with a spectrophotometer. (Refer to Appendix A.)
2. Dilute the aRNA sample(s) with nuclease-free water. Each gel well can be loaded with 1–3 µg of aRNA.
3. Prepare aRNA gel sample by mixing 6 µL of diluted aRNA with 6 µL of 2X Gel Loading Buffer.
4. Incubate for 3–5 minutes at 65°C. Cool on ice.
5. Prepare 1X RNA MOPS Running Buffer and fill gel electrophoresis unit. Place agarose gel into the unit.
6. Load 12 µL of sample per well of the agarose gel. Include RNA Ladder in one or more lanes.
7. Electrophorese at 5–7 Volts per centimeter.
8. Stain the gel with SYBR® Gold Nucleic Acid Gel Stain for 30 minutes or according to the protocol supplied with the reagent. Alternatively, stain with Ethidium Bromide (0.5–1.0 µg/mL).
9. Visualize the gel on a UV transilluminator.

The bulk of the aRNA product is 250 to 1800 bases in length after one round of amplification, and ranges from under 200 to over 600 bases after two rounds of amplification.

F. Generation of Labeled aRNA for GeneChip® Probe Arrays

GeneChip® Probe Arrays (Affymetrix) require using biotin-labeled aRNA. The RiboAmp Kit can be coupled to the ENZO® BioArray™ High Yield™ RNA Transcript Labeling Kit (T7) (Affymetrix Cat. # 900182) *for nucleic acid arrays* to yield suitable RNA sample for hybridizing to a GeneChip®. If you use this approach, the ENZO® Kit reagents and protocol are substituted during the IVT reaction of the RiboAmp Kit protocol. Read the ENZO® Kit instruction manual thoroughly. Labeled aRNA is subsequently purified utilizing RiboAmp Kit reagents with a modified protocol.

1. Perform Round One of amplification according to the RiboAmp RNA Amplification Kit protocol starting from 10–30 ng of total cellular RNA or 500–2000 LCM cells.
2. Perform Round Two of amplification through cDNA Purification according to the RiboAmp Kit protocol.
3. Perform RNA transcript labeling according to the protocol of the ENZO® BioArray™ High Yield™ RNA Transcript Labeling Kit (T7) using the sample (from step #2) as the cDNA template. Adjust the final volume of the cDNA sample by vacuum concentration or with RNase-free water provided in the RiboAmp Kit, as necessary.
5. Proceed to Labeled aRNA Purification or store at –20°C.

⚠ *Do not allow sample to dry completely in the vacuum centrifuge! Quantitative recovery of material may be severely compromised.*

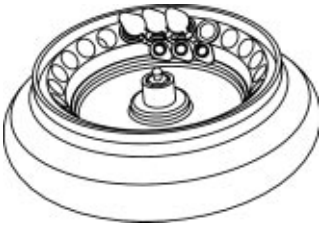
Labeled aRNA Purification

1. Add 250 µL of RNA Binding Buffer (RB) to a new DNA/RNA Purification Column and hold for 5 minutes at room temperature. Centrifuge at 16,000 x *g* for 1 minute.
2. Add 200 µL of RNA Binding Buffer (RB) to the IVT Reaction sample and mix thoroughly. Pipette the entire sample volume into the purification column.

⚠ *RNA Binding Buffer (RB) must be at room temperature and thoroughly mixed before use. A precipitate may form during long-term storage. Dissolve precipitate by mixing. If necessary, warm the RB vial to redissolve.*

3. To bind the aRNA to the columns, centrifuge at 100 x *g* for two minutes (or lowest speed setting), immediately followed by centrifugation at 10,000 x *g* for 30 seconds.
4. Add 200 µL of RNA Wash Buffer (RW) to the purification column and centrifuge at 10,000 x *g* for 1 minute.
5. Add 200 µL of fresh RNA Wash Buffer (RW) to the column, and centrifuge at 16,000 x *g* for 2 minutes.
6. Discard the flowthrough and used collection tube.
7. Place purification column onto a new 0.5 mL centrifuge tube and carefully add 30 µL of RNA Elution Buffer (RE) directly onto the center of the purification column membrane (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of RE into the membrane). Gently tap the purification column to distribute the buffer if necessary
8. Place the assembly into the centrifuge as shown, and centrifuge at 1,000 x *g* for 1 minute and then at 16,000 x *g* for one minute. Discard the column and retain the flowthrough containing aRNA in the microcentrifuge tube for further processing.
9. Measure the O.D. of the product at A_{260} and A_{280}. To achieve an appropriate dilution for measurement, remove a 1–5 µL aliquot and dilute with distilled water to 100 µL.
10. The biotinylated aRNA is ready for fragmentation and hybridization according to the GeneChip® Probe Array protocols. Refer to the Affymetrix protocol.

⚠ *Avoid splashing flowthrough in the collection tube onto the purification column. If flowthrough waste liquid wets the outside of the column, recentrifuge the column at 16,000 x g to remove the liquid.*



⚠ *To avoid potential breakage of the microcentrifuge tube cap during centrifugation, insert the purification tube/0.5 mL tube assembly into a lidless 1.7/2.0 mL tube. Insert this assembly into adjacent rotor holes as ilustrated. Rest the tube cap against the tube immediately clockwise to it. Place an empty, lidless 1.7/2.0 mL tube into the rotor hole adjacent in the clockwise direction to the last assembly.*

Arcturus
400 Logue Avenue
Mountain View, CA
USA 94043
www.arctur.com

888.446.7911
650.962.3020 tel
650.962.3039 fax
contact@arctur.com

Arcturus GmbH
Germany
[49] (0) 61 05-40 88 22 tel
[49] (0) 61 05-40 88 40 fax
europecontact@arctur.com

Arcturus Ltd.
United Kingdom
[44] (158) 246-9010 tel
[44] (158) 246-7988 fax
sales@arctur.co.uk

Arcturus SARL
France
[33] (1) 300-75 979 tel
[33] (1) 300-75 651 fax
europecontact@arctur.com

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